

**Research Article****Evaluation of anti-inflammatory and antinociceptive effects of latex from *Pergularia daemia* in experimental animal models**Nandhakumar Palraj<sup>1</sup>, Vairathevar Sivasamy Vasantha<sup>1\*</sup>, Natarajan Chidambaranathan<sup>2\*</sup>, Muthunanthevar Vijayan<sup>3</sup><sup>1</sup>Department of Natural Products Chemistry, Madurai Kamaraj University, Madurai, Tamilnadu, India<sup>2</sup>K.M. College of Pharmacy, Uthangudi, Madurai, Tamilnadu, India<sup>3</sup>Central Electrochemical Research Institute, Functional Materials Division, Karaikudi, Tamilnadu, India

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**Abstract**

**Background:** *Pergularia daemia* (Apocynaceae) plays an important role in traditional ayurvedic and siddha for the treatment of asthma, bronchitis, and jaundice. Interestingly, the latex of *Pergularia daemia* (PDL) is applied to cure headache, migraine, boils, wounds and scabies. **Objective:** Due to the pain relieving potential of PDL, the present study is aimed to evaluate the different phases of anti-inflammatory and antinociceptive efficacy of PDL in rat and mice models at very first time. **Material and Methods:** The important and major phytoconstituents of methanolic latex extract was quantified by using GC-MS and HPLC-DAD techniques. Orally, pre-treated three different doses (50, 100, 200 mg/kg) of PDL in order to evaluate distinct phases of anti-inflammatory and antinociceptive effects. **Results:** The inhibition ratio of the PDL (200 mg/kg) on carrageenan, dextran-induced paw oedema, xylene-induced ear oedema and cotton pellet granuloma in rats were 59.2%, 51.0%, 53.1% and 42.5%, better than the inhibition ratio of indomethacin (5 mg/kg) were 49.5%, 50%, 44.4% and 37.6%. At 200 mg/kg, the PDL reduces writhing to 52.3% in acetic acid-induced abdominal constriction model, paw licking to 45.65% in formalin test and increases the response latency to 25.24% in hot plate test. In particular, Naloxone reversed the antinociceptive potential of PDL and Morphine in both phases of formalin and hot plate tests which suggests PDL activates opioid receptor and blocks the release of glutamate and substance P. **Conclusion:** PDL sustains exemplary anti-inflammatory and antinociceptive activity along with peripheral and central analgesic properties which mediated through the activation of opioid receptor.

**Keywords:** *Pergularia daemia*; Apocynaceae; anti-inflammatory; antinociceptive; Opioid system

**Introduction**

Inflammation is an intricate pathophysiological process mediated by array of signalling molecules produced by leukocytes, macrophages and mast cells response of living tissue to undesirable stimuli. The displacement of leukocytes from blood to tissue leads plasma protein extravasation at inflammatory site and Macrophages release NO, Prostaglandins and proinflammatory mediators (TNF- $\alpha$ , IL-6, IL-1B) (Gokhale et al., 2002; White, 1999). Mast cells secrete numerous vasoactive peptides and pro-inflammatory mediators such as

histamine, serotonin, TNF, kinins (Julius & Basbaum, 2001). These pro inflammatory mediators cause severe pain has become a complicated field. The known compounds from natural pharmacophores and synthetic compounds still struggle with side effects. Unfortunately, side effects such as gastric lesions caused by NSAIDs and tolerance and dependence induced by opiates, the use of these drugs as anti-inflammatory and analgesic agents have not been successful in all the cases. As a result, promising new anti-inflammatory and analgesic drugs are seeking as a replacement to NSAIDs and opiates (Dharmasiri et al., 2003).

*Pergularia daemia* (forsk.) is a perennial herb which belonging to family Apocynaceae, are growing extensively along shrub areas of tropical and subtropical regions in India (Karthishwaran & Mirunalini, 2010). *Pergularia daemia* has long been engaged in traditional ayurvedic and siddha

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medicine in folk remedies for treating head, ear, eye and tooth ache (Bhaskar & Balakrishnan, 2009). Precisely the latex is practiced to cure Migraine, boils, Lymph glands, ringworm, scabies and leucoderma (Rao & Henry, 1996; Sen et al., 2010). Leaf juice and latex are used in diarrhoea, asthma and applied in rheumatic swellings (Bhaskar, 2006). Till now, most researches on *Pergularia daemia* focus multiple pharmacological properties and characterise the phytochemicals derived from various parts of the plant unless latex (Karthishwaran & Mirunalini, 2010). In this study, because of antimigraine and pain relieving efficacy of latex from *Pergularia daemia* (PDL), was primarily directed to evaluate the different models of anti-inflammatory and anti-nociceptive activities in vivo.

## Materials and methods

### Experimental animals

Male and female Swiss albino mice (25-30 g) and Wistar rats (250-280 g) were used. Animals were maintained under standard conditions (i.e. at  $26 \pm 2^\circ\text{C}$ , humidity: 45-50% and 12 h natural light/dark cycle) and fed with standard pellet diet and distilled water ad libitum. Each of these treatment groups were classified as six animals/ group. The protocol of the study was approved by the Institutional Animal Ethics Committee (IAEC) of K.M. College of Pharmacy, Madurai, constituted under Committee for Purpose of Control and Supervision of Experiments on Animals (CPCSEA), (approval No IAEC/KMCP/41/2018).

### Plant identification and latex collection

Latex of *Pergularia daemia* was collected from around the areas of Thiruvathavur village, Melur Taluk, Madurai district, Tamilnadu, India. The plant identification and authentication was done by Dr. C. Murugan, Scientist 'D' & Head, Botanical survey of India, Coimbatore. A voucher specimen (BSI/SRC/5/23/2018/TECH/1674) was deposited at the Herbarium of the Department of Natural products chemistry, School of chemistry, Madurai Kamaraj University, Madurai. The collected latex (500g) was dried in oven at  $35^\circ\text{C}$  to obtain 30% dry powder.

### Preparation of extract

The semidried latex powder 125 g was extracted with 90% aqueous methanol for 3 h. The extracts were filtered and concentrated by using rota vapour and lyophilized to yield the crude (11 g). The yield of the extract 24.1% was screened for active phytochemical constituents using standard protocol indicated the presence of alkaloids, flavonoids, steroids, terpenoids and phenolic compounds (Harborne, 1998).

### GC-MS analysis of PDL

GC-MS investigation of PDL was executed using Agilent 7820A (Agilent Technologies) coupled with MSD quadrupole detector 5977E (Agilent Technologies). Isolation of analytically

gas chromatography was accomplished by using the Hewlett Packard HP-5MS (Ultra inert) silica capillary column ( $30\text{ m} \times 250\ \mu\text{m} \times 0.25\ \mu\text{m}$ ). For GC-MS detection, an electron ionization system with ionizing energy of 70eV was used. Helium gas (99.999%) was used as the carrier gas at constant flow rate 1 ml/min and an injection volume of 1L was engaged (split ratio of 1:10); injector temperature  $250^\circ\text{C}$ ; ion-source temperature  $280^\circ\text{C}$ . The total run time was 23.50 min. The relative % amount of each component was measured by comparing its average peak area to the total areas. The identification of compounds and interpretation on mass spectrum GC-MS was conducted using the database of National Institute Standard and technology (NIST) having more than 62,000 patterns.

### Acute oral toxicity study

As stated by OECD guidelines-425 (OECD, 2008), Wistar rats were classified into five groups of six each. Group I was standard control served with distilled water. From groups II to IV were orally treated with PDL in different doses of 50, 300 and 2000 mg/kg. After the 14 days of observation period, the animals were euthanized and histological studies were conducted on kidney of the animals using haematoxylin – eosin (H&E) staining. Photomicrographs were taken with light microscope (Lab vision I-3000) at 40X.

### Carrageenan-induced paw oedema (an acute inflammation model)

The male rats were partitioned into five groups of six each. The control group I administered with 10 mL/kg distilled water. Group II served as positive control dosed with Indomethacin (5 mg/kg) administered via p.o. 1 h before the carrageenan injection (Dharmasiri et al., 2003). The Groups III–V were dosed p.o. with 50, 100 and 200 mg/kg of PDL dissolved in water after 1 h of the administration of doses, 0.1 mL of 1% carrageenan in saline was injected into the subplantar region in the left hind paw of rats. Paw volumes were determined by using Plethysmometer (Ugo Basil, Italy) at times 2 and 4 h post carrageenan administration.

### Dextran-induced Paw Oedema (a sub-acute inflammation model)

The rats were served as described above and the oedema was induced in the right hind paw by subplantar injection of 0.1 mL of freshly prepared 1% dextran solution (Motoyama et al., 2016) and paw volume was calculated 30 min before and after dextran injection.

### Cotton Pellet-induced Granuloma (a chronic inflammation model)

Thirty minutes after the administration of drug/ vehicle, the animals were anesthetized with diethyl ether and a sterile

cotton weighing ( $25 \pm 1$  mg) impregnated with saline was implanted subcutaneously in the ventral region (Kumar et al., 2016). The rats were divided into five groups. Group I served as control administered with 10 mL/kg of distilled water and group II served as positive control dosed with 5 mg/kg of indomethacin. The Groups III–V was served for 6 days at the doses of 50, 100 and 200 mg/kg of PDL p.o. On 7<sup>th</sup> day, the terminal blood was collected and the cotton pellets were eradicated. The animals were anesthetized again and the cotton pellets were ejected surgically and made free from extraneous tissues. Pellets were abolished and dried at 60 °C until the weight remained constant and the net dry weight of the granuloma tissue was calculated.

#### **Xylene-induced mouse ear oedema (an inflammation model)**

Adult male mice assigned to five groups of six animals. Group I served as control administered with 10 mL/kg of distilled water. Group II served as positive control dosed with 5 mg/kg indomethacin 1 h before the xylene injection. Groups III–V was served with 50, 100 and 200 mg/kg of PDL. The inflammation was induced by the topical application of 30  $\mu$ L/ear of xylene on the anterior and posterior surfaces of the right earlobe. One hour later the animals were euthanized and two ear punches (5 mm diameter) were taken from each mouse and weighed the swelling induced by xylene which was assessed as the increase in weight of ear punch of served groups over unserved one indicated the oedema index (Sadeghi et al., 2014).

#### **Acetic acid-induced writhing test (pain model)**

To estimate analgesic activity of the plant extract, this method was described by Gupta et al., (Gupta et al., 2015). Adult male mice were classified into five groups of six each. Group I served as control administered with 10 mL/kg of distilled water. Group II classified as positive control dosed with 100 mg/kg ASA p.o. (Park et al., 2005) 30 min before induction. Groups III–V was administered with 50, 100, and 200 mg/kg of PDL p.o. Thirty minutes later, 10 mL/kg of acetic acid (0.6%) was injected to all the animals in diverse groups. The number of abdominal writhing (constrictions) occurring between 5 to 20 min after acetic acid was estimated. The remarkable reduction of writhes in tested animals correlated to those in the control group was examined as an antinociceptive response.

#### **Hot plate test (pain model)**

The animals were divided into six animals of five groups. Group I served as control treated with 10 mL/kg of distilled water. Group II served as positive control and was dosed with morphine (5 mg/kg) administered for 1 h before hot plate test. Group III–V was treated with 50, 100 and 200 mg/kg of PDL p.o. The indisposition time (licking paws or jumping) was registered as response latency. The abort time for the reaction was 15 seconds. The effects of Naloxone on rats along with PDL were

divided into two groups of 6 each. First group received 200 mg/kg of PDL during other group treated with Naloxone (5 mg/kg). In both cases, hot plate latencies were calculated at every 60 min after oral administration of tested samples (Eddy & Leimbach, 1953).

#### **Formalin test**

Pain was induced by injecting 20  $\mu$ L of 2.5% formalin (0.92% of formaldehyde) in distilled water in the subplantar of the right hindpaw (De Sa et al., 2012). Rats (six per group) were given PDL (50, 100 and 200 mg/kg p.o.), indomethacin (5 mg/kg, i.p), and distilled water (p.o.) 30 min prior to injecting formalin. The behavioural nociceptive response including biting, licking and scratching of the injected paw were noted. The time spent was recorded up to 30 min. Generally formalin induced test is a biphasic system; early phase is 0–5 min and 15–30 min considered as late phase of nociceptive response. So as to examine the involvement of opioid system in antinociceptive action of the PDL, the rats were treated with Naloxone (5 mg/kg). After 15 min, the PDL (200 mg/kg p.o.) administered into the animals and the nociceptive response was determined 30 min hereafter.

#### **Statistical analysis**

Results were expressed as mean  $\pm$  SEM. The statistical significance was determined by one-way analysis of variance (ANOVA) followed by Dunnett's and Tukey's multiple comparison tests. Values less than 0.001 ( $p < 0.001$ ) and 0.05 ( $P < 0.05$ ) were considered as indicative of significance. The  $IC_{50}$  values were calculated from the dose/response nonlinear regression plots by using GraphPad prism 5.0.

#### **Results**

##### **GC-MS analysis of PDL**

A total of 10 major peaks were identified from PDL in GC-MS (Figure 1) with the major compounds constituted of 9-octadecenamide, hexadecanamide and myristamide (42.1%) n-Hexadecanoic acid (12.6%), Octadecanoic acid (11.7%), 2-Methoxy-4-vinylphenol (10.9%), 2,3-dihydro-3,5-dihydroxy-6-methyl pyranone (5.5%), Oleic acid (9.75%), methyl-d-galactopyranoside (6.8%), methyl-d-glucopyranoside (3.9%), 2,3-dihydro-benzofuran (3.5%), 1,4- vinylphenyl acetate (3.4%), 4-Hydroxy-2-methylacetophenone (19.8%), 4-Hydroxy-3-methylacetophenone (14.8%), l-Gala-l-ido-octose (3.1%), and 2-Dodecenoic acid (3.82%).

##### **Acute toxicity study**

PDL at the dose of 200 mg/kg (Figure 2a) shows normal cortex and medulla of kidney. The parts of the nephron

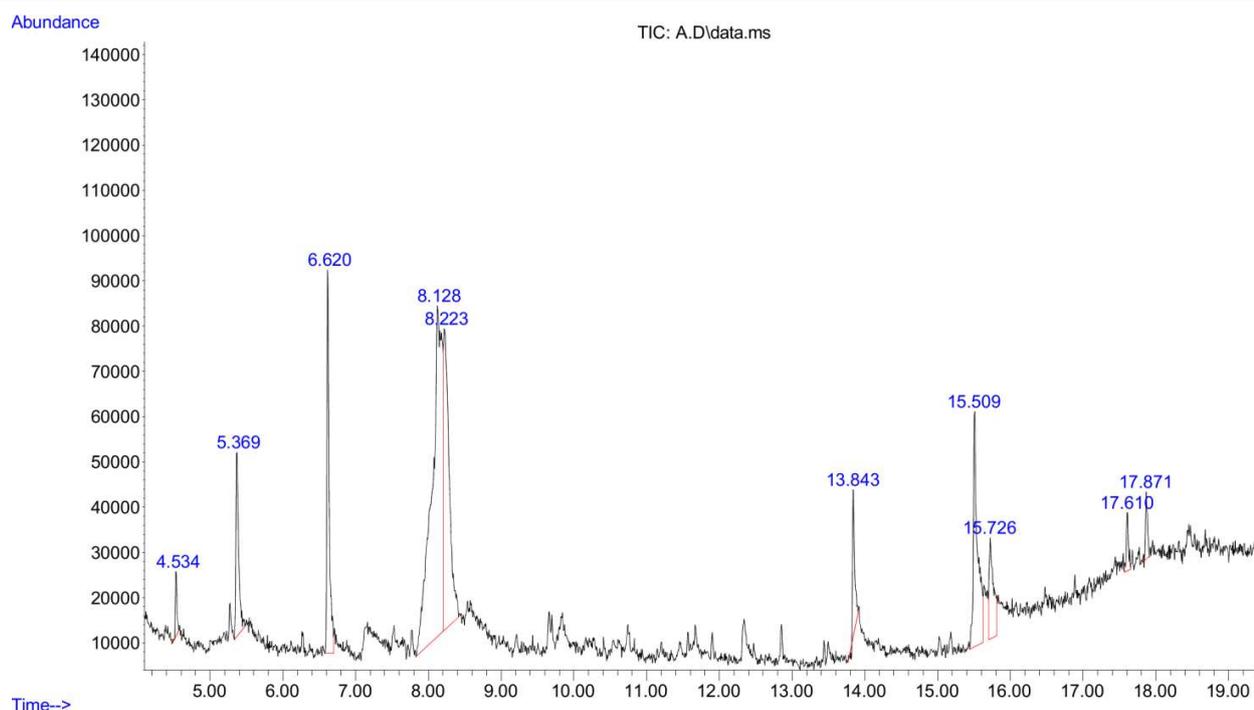


Figure 1. The GC-MS chromatogram of methanolic extract of PDL

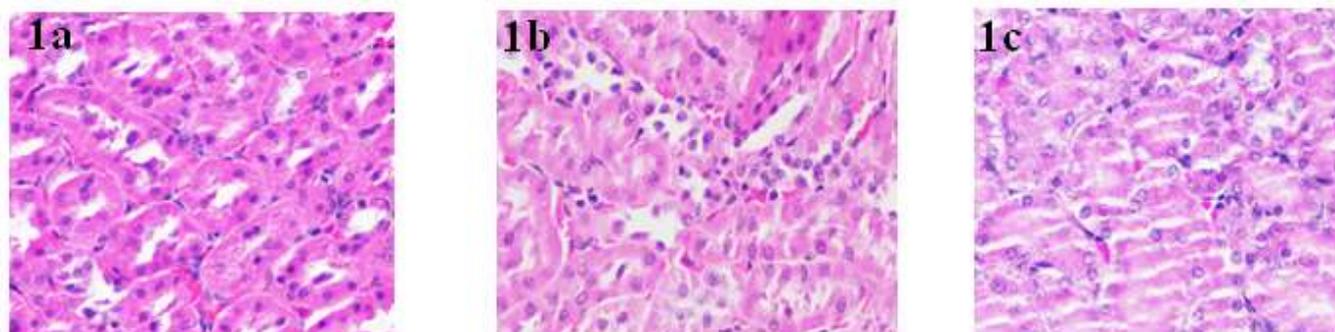


Figure 2. The Histopathological images of PDL (1a (200mg/kg) 1b (2000mg/kg) 1c (Toxic group)) ingested mice kidney

namely proximal and distal convoluted tubule was found to be normal. Both tubules possess a distinct lumen. This group exhibited normal architecture of kidney. While, high dose of 2000mg/kg (Figure 2b) shows normal cortex and medulla and lumen of the tubules shows moderate distortion with enlarged nuclei of simple squamous epithelium and normal cyto architecture was found. Moreover, the toxic group (Figure 2c) altered the cortex and medulla of the nephrons differentiation was absent. Lumen and structure of the tubules were severely damaged due to toxicity.

#### Carrageenan-induced paw oedema test

Figure 3 describes the efficacy of PDL for the inhibition of swelling induced by carrageenan is dose-dependent at 2 h and 4 h when compare with control. PDL at 200 mg/kg possess 62.77% of inhibition at 2 h after carrageenan injection which is greater than 57.97% of indomethacin (5 mg/kg). The  $IC_{50}$  values for the restrain of paw oedema by PDL were 176.5 mg/kg and 134.6

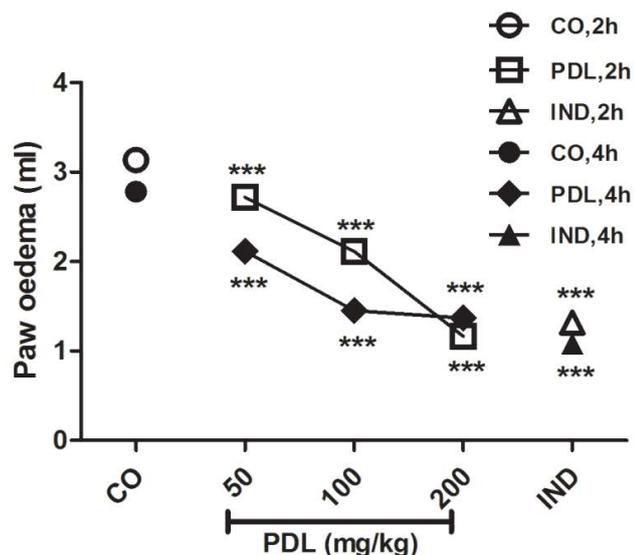
mg/kg for 2 h and 4 h, respectively.

#### Dextran-induced rat paw oedema

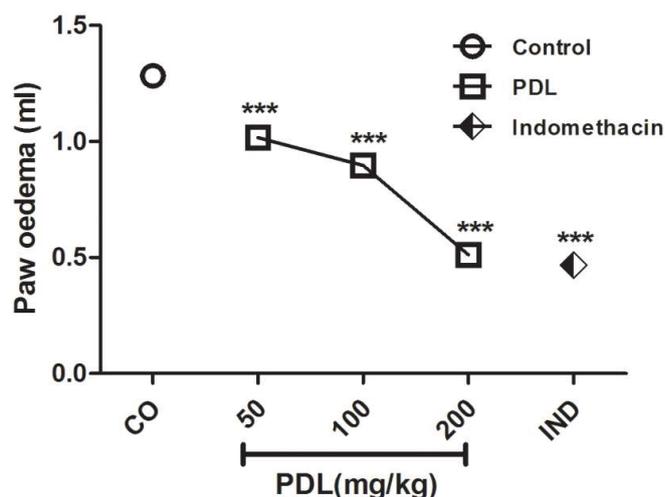
The results (Figure 4) represents the suppression of oedema caused by dextran at a dose of about 200 mg/kg of PDL which has shown somewhat similar inhibition potential 60.15%, against Indomethacin (63.89%) (5 mg/kg). The  $IC_{50}$  value for the suppression of paw oedema by PDL is 170.5 mg/kg.

#### Cotton pellet granuloma in rats

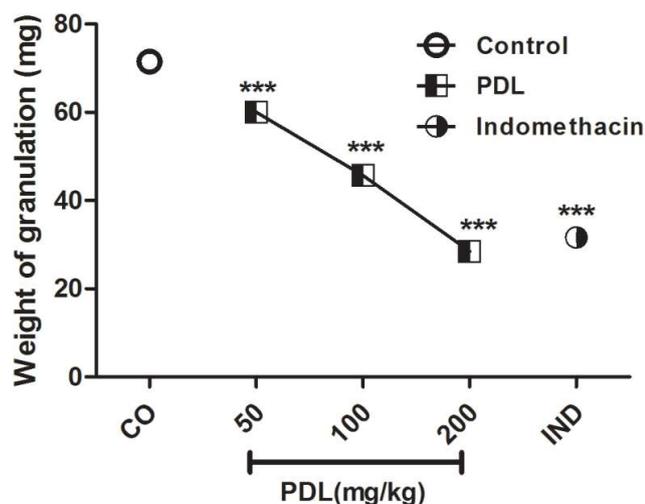
The results from the Figure 5 exhibit that PDL possesses significant dose-dependent depression activity on cotton pellet granuloma and expressive inhibition of 62.93% at the dose of 200 mg/kg which is superior to inhibitive activity (60.13%) of indomethacin (5 mg/kg). The deduced  $IC_{50}$  value for the depression of granuloma by PDL is 140.7 mg/kg.



**Figure 3.** Effects of PDL at the dose 50-200 mg/kg and Indomethacin (IND) at the dose of 5 mg/kg on Carrageenan-induced rat paw oedema at 2 h and 4 h intervals. CO: control group. Values are mean  $\pm$  SEM (n = 6). \*\*\*P < 0.001, asterisks denote the significance levels compared with the control group (one-way ANOVA, followed by Dunnett's post hoc test)



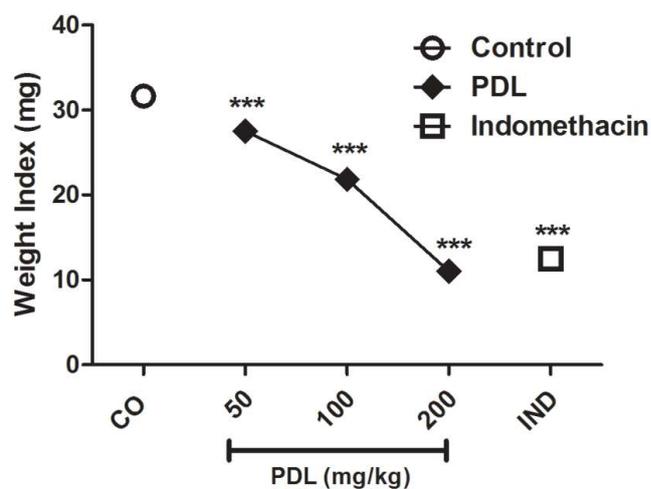
**Figure 4.** Effects of PDL at the dose 50-200 mg/kg and Indomethacin (IND) at the dose of 5 mg/kg on Dextran-induced rat paw oedema. CO: control group. Values are mean  $\pm$  SEM (n = 6). \*\*\*P < 0.001, asterisks denote the significance levels compared with the control group (one-way ANOVA, followed by Dunnett's post hoc test)



**Figure 5.** Effects of PDL at the dose 50-200 mg/kg and Indomethacin (IND) at the dose of 5 mg/kg on cotton-pellet granuloma in rats. CO: control group. Values are mean  $\pm$  SEM (n = 6). \*\*\*P < 0.001, asterisks denote the significance levels compared with the control group (one-way ANOVA, followed by Dunnett's post hoc test)

#### Xylene-induced ear oedema

The efficacy of PDL on xylene induced mouse ear oedema is represented in Figure 6 PDL reduce remarkably the xylene induced mouse ear oedema when compared to the control and produces a dose-dependent decrease in response at 200 mg/kg and PDL exhibits inhibition of 65.18% which better than the inhibition activity (60.44%) of indomethacin (5 mg/kg). The

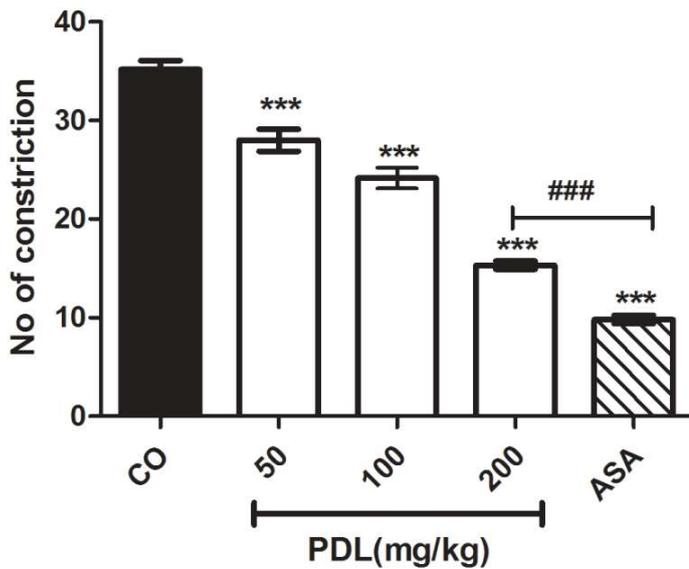


**Figure 6.** Effects of PDL at the dose 50-200 mg/kg and Indomethacin (IND) at the dose of 5 mg/kg on xylene-induced mouse ear oedema. CO: control group. Values are mean  $\pm$  SEM (n = 6). \*\*\*P < 0.001, asterisks denote the significance levels compared with the control group (one-way ANOVA, followed by Dunnett's post hoc test)

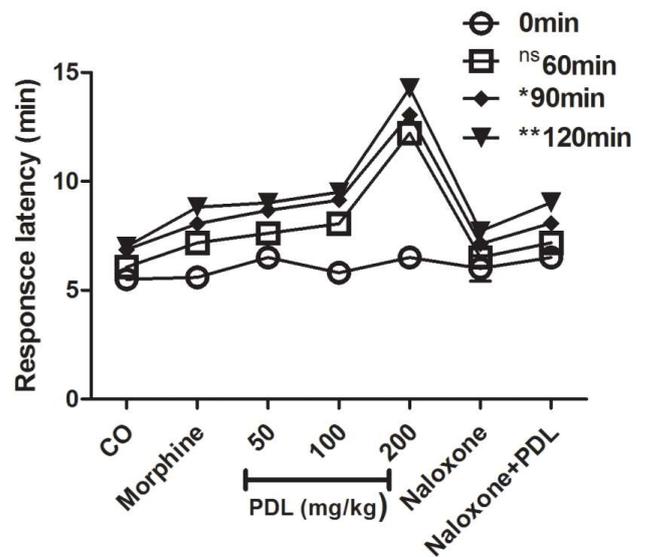
derived  $IC_{50}$  value for the depression of ear oedema by PDL is 169 mg/kg.

#### Acetic acid-induced writhing

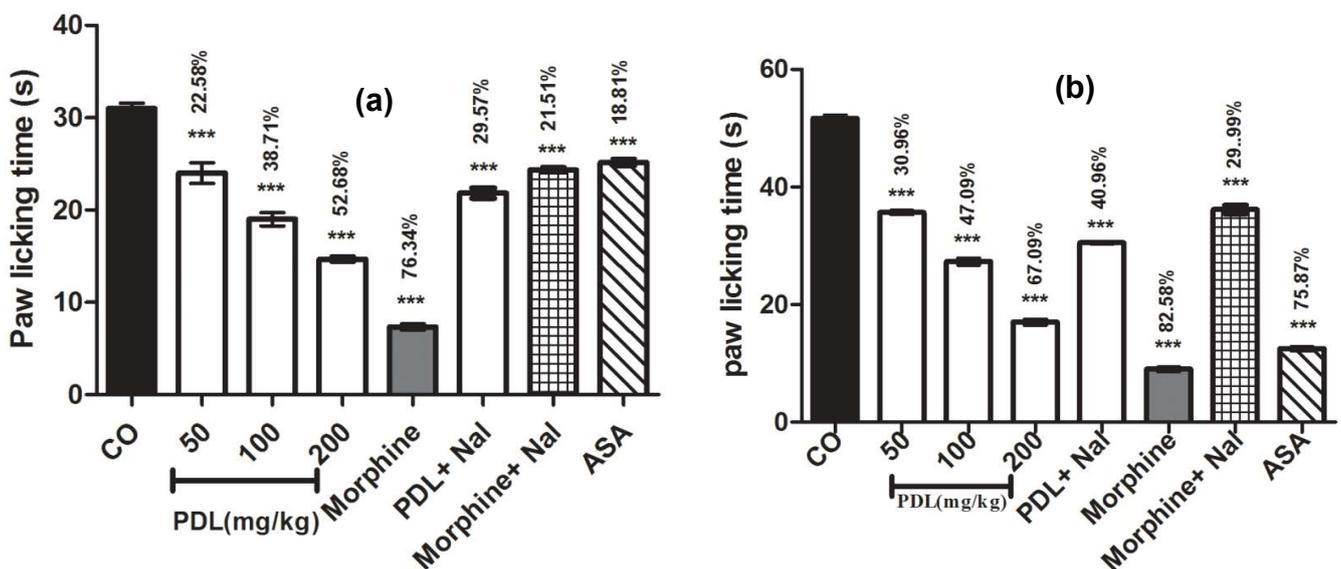
The passage of writhing experience induced by acetic acid (0.6%) in PDL is summarized in Figure 7. PDL displays significant analgesic activity like dose-dependent manner and the influence is 56.39% at the dose of 200 mg/kg, lesser



**Figure 7.** Effects of PDL at the dose 50-200 mg/kg and Aspirin (ASA) at the dose of 50 mg/kg on acetic acid- induced writhing in mice. CO: control group. Values are mean  $\pm$  SEM (n = 6). \*\*\*P < 0.001, asterisks denote the significance levels compared with the control group, (One-way ANOVA, followed by Dunnett's post hoc test): ###P < 0.001 when compared to Positive control ASA. (One-way ANOVA, followed by Tukey's Multiple Comparison Test).



**Figure 8.** Hot plate used antinociceptive activities of PDL at the dose 50-200 mg/kg. CO: control group. Values are mean  $\pm$  SEM in seconds (n = 6). \*P < 0.05, asterisks denote the significance levels compared with the 0min response (one-way ANOVA, followed by Dunnett's post hoc test).



**Figure 9.** Formalin induced antinociceptive activities of PDL at the dose 50-200 mg/kg in a) first phase b) second phase. CO: control group. Values are mean  $\pm$  SEM (n = 6). \*\*\*P < 0.001, asterisks denote the significance levels compared with the control group (one-way ANOVA, followed by Dunnett's post hoc test).

than the activity (72.03%) of ASA (100 mg/kg). The  $IC_{50}$  value for the inhibition of constriction response by PDL is 159 mg/kg.

#### Hot plate test

Figure 8 exhibits the results of the hot plate test. PDL influences the latency time in a dose-dependent manner. At 200 mg/kg dose,

PDL significantly increases the response latency (35.71%) at 2 h. Meanwhile, Morphine (5 mg/kg) significantly increases the response latency time with a maximum protective effect of 104% at 2 h. The  $IC_{50}$  value for the increased the response latency by PDL is 101.4 mg/kg for 2 h.

### Formalin test

Intraplantar injection of 2.5% of formalin evokes a characteristic biphasic licking response. Pre-treatment with different doses (50, 100 and 200 mg/kg, p.o.) of PDL shows a significant and dose-dependent effect against the duration of licking activity in both phases (Figure 9 a&b). Particularly, morphine (5 mg/kg, i.p.) produced marked inhibition ( $p < 0.001$ ) of both the neurogenic pain (early phase, 76.34%) and inflammatory pain (late phase, 82.58%) of the formalin test. In contrast, the treatment of animals with ASA (100 mg/kg, p.o.) causes significant inhibition (75.87%) of the late phase, but not in the early phase (18.81%). On the other hand, Naloxone reverses significantly the antinociceptive effect of PDL (200 mg/kg) and morphine in both phases ( $p < 0.001$ ) of the formalin test. The  $IC_{50}$  values are 167.7 mg/kg and 176.2 mg/kg for both first and second phases, respectively.

### Discussion

In the acute oral toxicity test dose of 2000 mg/kg of PDL did not produce mortality in mice during 14 days observation. The mice did not exhibit any signs of toxicity, aberrant behaviour or other physiological activities.

The results of the present study exhibited that PDL has an ability of free radical scavenging activity against DPPH and FRAP (Figure S1&S2) which is highly related to the presence of hydroxyl groups of a diverse group of plant polyphenolic compounds, such as flavonoids and phenolic acids. The total phenolic and flavonoid content is found to be  $21.44 \pm 0.2102 \mu\text{g GAE/g}$  and  $50.6 \pm 1.83 \mu\text{g QE/g}$  respectively (Figure S3&S4). The flavonoids have redox properties, which admit them to act as antioxidant and which depends on the occupancy of positions of free OH groups, particularly 3-OH (Bendary et al., 2013). The inducement of NMDA pain signals is highly influenced by the free radicals (Bodhinathan et al., 2010). Therefore, PDL's free radical scavenging activity could be played major role in pain inhibition.

Moreover, PDL demonstrated that have dose-dependent activities against inflammation as revealed in the carrageenan-induced paw oedema, dextran-induced rat paw oedema, cotton pellet granuloma, xylene-induced ear oedema besides, acetic acid induced writhing, hot plate and formalin tests declare that PDL overwhelm acute pain.

The carrageenan induced acute inflammation is believed to be bi-phasic (Dharmasiri et al., 2003). The initial phase (1-2 h) is mediated by the early release of histamine and serotonin followed by the release of kinins and extensively releases bradykinin, prostaglandins (PGs) and lysosome. The later phase is proclaimed to be sensitive to both steroidal and nonsteroidal anti-inflammatory agents clinically (Chen-Xiao et al., 2011). The present study revealed that PDL displayed remarkable anti-inflammatory activity and possessed greater inhibition ratio at 4

h (55%) than 2 h (52%). The PDL influences the both phases of inflammation caused by the suppression of PGs synthesis inhibition, antihistamine and serotonin activities which are stimulate nociceptors and thus induce pain. The inhibitory effect of PDL on carrageenan induces inflammation may be due to inflammatory mediators.

The cotton pellet induced granuloma model is believed to assess transudative, exudative and a proliferative phase of sub-acute inflammation. The first and second phase involves inhibition and exudation of fluid containing the low protein and finally emergence of collagen, mucopolysaccharide synthesis and increase the number of fibroblasts around the cotton pellets (Panthong et al., 2003). After 7 days, the cotton pellets are removed, dried and weighed. PDL remarkably reduced the final dry weight of the cotton pellets, i.e. it decreased the amount of newly formed connective granulomatous tissue, indicated that the potential of reducing the synthesis of mucopolysaccharides and collagen and the number of fibroblasts. PDL decreases the weight of granuloma tissue in a dose-dependent manner, confirming its efficacy in the chronic phase of inflammation.

From xylene-induced mouse, ear oedema is used to determine the levels of vasodilatation and plasma protein extravasations of neurogenic inflammation (Li et al., 2011). From this study, PDL (200 mg/kg) significantly decreases the ear oedema in a dose-dependent manner. The inhibition of ear oedema indicated that PDL enervated vasodilatations and plasma extravasations of neurogenic inflammation, which were crucial in restraining factors of the early stage of acute inflammation. Dextran that induces anaphylactic reaction which is characterized by extravasation and oedema formation, as a consequence of liberation of histamine and serotonin (Saleem et al., 2017) which is also significantly suppressed by PDL. Therefore, the anti-inflammatory potential of PDL could be by cause of antihistamine or antiserotonin nature and the inhibitory activity of PDL is shown somewhat better efficiency against standard indomethacin.

From this result, it is suggested that PDL generate anti-inflammatory action via inhibiting the inflammatory mediators of the acute phase of inflammation. As a concomitant reaction, the oedema forms caused by fluid and plasma proteins are extravasated. The analgesic and nociceptive activities of PDL are determined by chemically and thermally by acetic acid induced writhing, formalin test and hot plate test in mice, which could extent response to different grades of noxious stimuli (Victor et al., 2004). Generally, the acetic-acid writhing test is very sensitive but non-specific which may lead to possible false positive results (Bendary et al., 2013). The results acquired from

acetic acid induced writhing are significant when PDL exhibits dose dependant response interaction at 50, 100 and 200 mg/kg, as writhing response of Indomethacin (5 mg/kg). The abdominal constriction induced by acetic acid has been attributed to the release of arachidonic acid which synthesizes prostaglandins via COX enzyme. As a consequence, the inhibition of prostaglandin synthesis is significantly an efficient antinociceptive mechanism in visceral pain. Since, PDL in this study shows very significant inhibition ( $P < 0.001$ ) (Figure 5) in acetic acid-induced pain, predicts as the analgesic effect produced by the PDL, for this reason the formalin and the hot-plate tests were employed.

The formalin and hot plate tests are more specific nociception model and employed to discriminate pain in its central and peripheral components (Mansouria et al., 2017). The administration of formalin into the hind paw of animals cause unique biphasic nociceptive licking and biting reply indicates early (neurogenic) and late phase (inflammatory pain) of inflammation. The early phase involves stimulation of nociceptors and second phase related with a release of inflammatory mediators such as prostaglandin, histamine, serotonin and bradykinin. Moreover, centrally acting drugs inhibit both phases fairly, while peripherally acting drugs like acetylsalicylic acid which block prostaglandins synthesis and inhibit the release of histamine, serotonin and bradykinin (Sulaiman et al., 2010). This present study exhibits that PDL in a dose-dependent manner significantly reduces the licking and biting event in both early and late phases when compare to control.

Moreover, the central analgesic effect of the PDL assists the results obtained from hot plate test, which has selectivity for opioid-derived analgesic drugs. Certainly, it is demonstrated that PDL exercises a remarkable extent in the response latency time to the thermal nociceptive stimulus, thus confirming the central activity of this extract.

Taking together, antiserotonin nature of PDL could activate 5-HT receptors (Kilinc et al., 2017) and suppression of neurogenic inflammation causes blockage of releasing substance p (Ramachandran, 2018) which justifies its antimigraine potential. Hence, PDL influenced not only anti-nociceptive but also anti-inflammatory activity. Besides, our results also acknowledged that pre-treatment with a nonselective opioid receptor antagonist, naloxone, reversed the antinociceptive effect of PDL and morphine in both phases of the formalin test as well as in the hot-plate test. These findings clearly implied that the antinociceptive effect of PDL was mediated by the activation of the opioid system and suggest that the analgesic efficacy of PDL may fall through opioid receptors. The incitation of opioid receptor decreases levels of cAMP, and obstructs the release of glutamate and substance P (Rusin et al., 1997). The blockage of pronociceptive or hyperalgesic substance may lead the analgesic activity (Menziés et al., 1998). Plant containing flavonoids (Kaempferol and Quercetin) and tannins possess significant analgesic activity and have been reported to inhibit the pro-inflammatory

cytokines like TNF- $\alpha$ , IL-6 (Kempuraj et al., 2005; Ramesh et al., 1998). Moreover, it has been found to interact with the opioid receptors (Maleki-Dizaji et al., 2007).

The data retrieved from HPLC-DAD analysis (Figure S5.1-S5.5) acknowledge the presence polyphenolics and flavonoids that may be accounted for these activities and may in part explain the mechanisms of its actions in this study. Polyphenolics (flavonoids) exert their antinociception via opioid receptor activation (Otuki et al., 2005; Rajendran et al., 2000) and Phenolic compounds such as prenylated phenolics inhibits production and release of inflammation-related cytokines; their effects on inhibition of cyclooxygenases and lipoxygenases (Brezani et al., 2018). From GC-MS analysis (Figure.1) it is revealed that the presence of pharmacologically active compounds of PDL contains approximately 42.1% of the constituents detected were fatty acid amide, namely 9-octadecenamide, hexadecanamide and myristamide. The present of fatty acid amide might contribute to the observed antinociceptive activity of PDL based on previous reports that several fatty acid amides hexadecanamide (Deciga-Campos et al., 2007) and derivatives of 9-octadecenamide demonstrated antinociceptive activity (Dray & Dickenson, 1991; Barriere et al., 2013). In specific, PDL's antinociceptive activity which are known for anti-inflammatory and analgesic activities due to inhibition of pro inflammatory mediators from COX-1 and COX-2 (Rang et al., 2007) in addition to, antioxidant and free radical scavenging ability (Kamat et al., 2000) which could possibly account for its anti-inflammatory action.

## Conclusion

In summary, these findings assist the role of PDL in traditional medicine as pain and inflammation drugs. Interestingly, the outcome of the present study advert that PDL possesses remarkable central and peripheral antinociceptive effects in animal models that are possibly mediated both by inhibition of pro inflammatory mediators generation and activation of an opioidergic mechanism. Moreover, the antinociceptive activity of PDL might be imputed to the presence of fatty acid amides and polyphenols (flavonoids) based compounds. Therefore, more precise studies are require to identify the bio active compounds which has the potential to exert an opioid antinociceptive activity at the central and peripheral level could be a good competitor for the evolution of new analgesic drug that is lack of dependence, tolerance and addiction effects as morphine.

## Conflict of interest

The authors declare no conflicts of interest

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### List of Abbreviations

OECD:	organisation for economic co-operation and development
NSAIDs:	nonsteroidal anti-inflammatory drugs
TNF $\alpha$ :	tumor necrosis factor alpha
IL:	interleukins
GC-MS:	gas chromatography-mass spectrometry
HPLC-DAD:	high performance liquid chromatography with diode array detection
DPPH:	1,1-diphenyl-2-picrylhydrazyl
FRAP:	ferric reducing antioxidant power
NMDA:	n-methyl-d-aspartate receptor
cAMP:	cyclic adenosine monophosphate
COX:	cyclooxygenase